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TITLE: Reversal of Doxorubicin Resistance in Human Breast Adenocarcinoma (MCF-7) Cells by Liposomal Monensin

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**13. ABSTRACT** *(Maximum 200 Words)*

The purpose of the present study was to overcome the doxorubicin resitance in human breast adenomacarcinom (MCF-7/dox) cells by the delivery of monensin in long-circulating (stealth) liposomes (SML). We have previously shown that SML could enhance the cytotoxicity of anticancer drugs. In order to increase the entrapment of monensin in SML, we modified our previous method by using pH-gradient method. In the present study, we studied the potential of SML (prepared by pH-gradient method) for their effect on the in-vitro cytotoxicity of anticancer drugs (doxorubicin, etoposide, paclitaxel) against both sensitive and resistant MCF-7 cells by crystal violet dye uptake assay. Further, the induction of apoptosis in resistant MCF-7 cells by the combination of doxorubicin with SML was also assessed by acridine orange staining and caspase-3 assay. Our results show that SML (10x10-8 M) enhance the in-vitro cytotoxicity of doxorubicin, etoposide and paclitaxel against sensitive MCF-7 cells by a factor of 5, 261 and 90, respectively. In case of resistant MCF-7 cells, there was 16.5, 5.6 and 2.8- fold potentiaiton of the cytotoxicity of doxorubicin, etoposide and paclitaxel, respectively by monensin liposomes (20x10-8 M). There was an enhanced apoptotic response (30%) in resistant MCF-7 cells treated with doxorubicin at 0.5 mcg/ml (1/50 th IC50 concentration for doxorubicin) with nontoxic concentration of monensin liposomes (20x10-8 M) in comparison to less than 10% apoptotic response observed in control, doxorubicin and liposomal monensin treated cells. The specific activity of caspase-3 in resistant MCF-7 cells treated with doxorubicin (2.5 mcg/ml) and monensin liposomes (20x10-8 M) was two times more than that of the cells treated with doxorubicin alone. The results indicate that it is possible to overcome the doxorubicin resistance in MCF-7 cells with liposomal monensin, which may be further explored in-vivo in nude mice with human breast tumor xenografts.

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## INTRODUCTION

Doxorubicin, a drug of choice for the treatment of breast cancer, has dose limiting cardiotoxicity and its repeated administration may lead to pleiotropic drug resistance in patients. Various agents such as verapamil have been used to overcome the doxorubicin resistance in various clinical studies unsuccessfully. We have previously shown that long-circulating (stealth) monensin liposomes (SML) could enhance the cytotoxicity of anticancer drugs (1,2). In order to increase the entrapment of monensin in SML, we modified our previous method by using pH-gradient method (3). The purpose of the present study was to overcome the doxorubicin resistance against human breast adenocarcinoma (MCF-7/dox) cells by SML and also to study the potential of SML in overcoming the drug resistance against other drugs such as paclitaxel and etoposide (VP-16) used in the treatment of human breast cancer. In-vitro cytotoxicity assay using crystal violet dye uptake was used to determine the reversal of drug resistance against both sensitive and resistant human breast tumor MCF-7 cells. Further, the induction of apoptosis in resistant MCF-7 cells by treatment with non-toxic concentrations of doxorubicin with SML was also assessed by acridine orange staining and caspase-3 assay.

## BODY

**Preparation of long-circulating (stealth) monensin liposomes (SML) by pH-gradient method:** Monensin liposomes were prepared using the following lipid composition based upon our previous study, dipalmitoylphosphatidylcholine: cholesterol: distearoyl glycerophosphoethanolamine-polyethylene glycol 2000: stearylamine, 10:5:1.4:1.4 (molar ratio). One hundred forty three milligrams of lipids in the above-mentioned ratio were taken in a pear-shaped flask and the lipids were dissolved in 20 ml of chloroform-methanol (2:1 v/v). The solvent was completely evaporated in a rotary evaporator to form a thin film of lipid. Multilamellar vesicles (MLVs) were obtained by shaking the flask (containing the lipid film) with 0.1 M HEPES buffer (pH 9.5). The MLVs were extruded using an Avestin Emulsiflex-C5 (Ottawa, ON, Canada) containing a series of polycarbonate membranes of gradually decreasing pore size (0.8, 0.6, 0.4, 0.2 and 0.1 μm) to produce small unilamellar vesicles (SUVs). Monensin, along with a tracer amount of radioactive $^{3}$H-monensin (10 μCi), was dissolved in ethyl alcohol-1mM hydrochloric acid (1:1 v/v) at 0.05% w/v and 0.2% w/v concentrations (pH 5.0-5.9). Monensin was entrapped in liposomes by adding 0.05% monensin solution (1, 2, 4, or 7.5 ml) or 0.2% monensin solution (1 ml) to SUVs (21 μmoles of phospholipid) and mixed for 30 minutes. Unentrapped monensin was removed by passing the SUVs through Sephadex™ G-25 M column. Subsequently, further purification of SUVs was performed by dialysis of SUVs against saline (Spectra/Por®, MWCO 12- 14,000, Spectrum Laboratories. Inc. Rancho Dominguez, CA, USA). The SUVs were then freeze-dried (FD 3.0, Appropriate Technical Resources, Inc., Laurel, MD, USA) employing trehalose and mannitol (each at 10% w/v) as lyoprotectants. SML containing radioactive $^{3}$H-monensin prepared as described above were used in all the subsequent experiments including the cytotoxicity and apoptosis studies.

**Characterization of SML for their particle size and monensin entrapment:**

Table 1: Entrapment efficiency and particle size data of SML prepared by pH-gradient method.

| Monensin concentration (%w/v) | Monensin solution used (ml) | Amount of monensin entrapped (mg) Mean±S.D. (n=4) | Entrapment efficiency (%) | Particle size after freeze-drying Mean±S.D. (n=4) |
|---|---|---|---|---|
| 0.05 | 1.0 | 0.057 (0.001) | 11.4 | 218 (2.9) |
| 0.05 | 2.0 | 0.112 (0.003) | 11.2 | 221 (10.4) |
| 0.05 | 4.0 | 0.267 (0.001) | 13.3 | 225 (5.5) |
| 0.05 | 7.5 | 0.366 (0.012) | 9.8 | 197 (4.0) |
| 0.2 | 1.0 | 0.28 (0.008) | 14.0 | 223 (6.5) |

The pharmacokinetics of SML in BALB/c mice is shown in Figure 1.

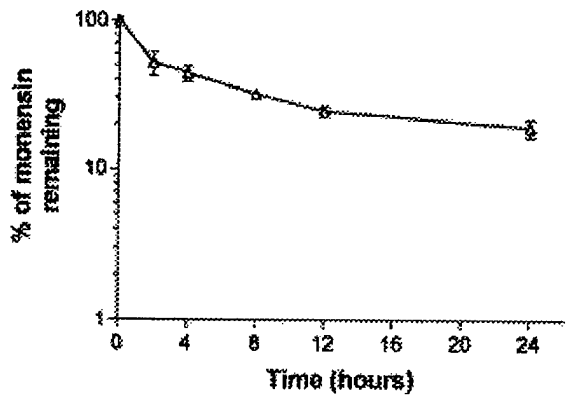



Figure 1: Pharmacokinetics of monensin in BALB/c mice upon intravenous administration of SML. The concentration of monensin in serum is plotted as a percentage of the injected dose.

**Potentiation of the cytotoxicity of doxorubicin, etoposide and paclitaxel against both sensitive and resistant MCF-7 cells by SML:** The effect of SML on the in-vitro cytotoxicity of DOX, ETP and PTX was studied by the crystal violet dye uptake assay. The results are given below in Table 2

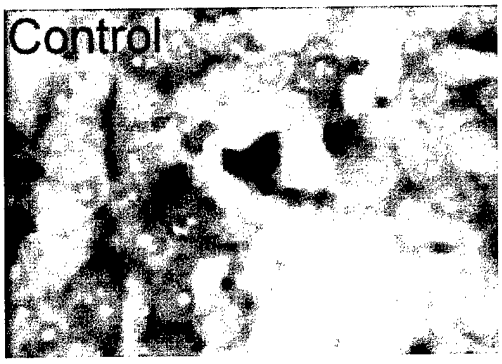
Table 2: Inhibitory concentration ($IC_{50}$) of anticancer drugs with or without SML against sensitive and resistant MCF-7 cells.

| Cell line | Drug | $IC_{50}$ for drug alone (μg/ml) | $IC_{50}$ for drug with SML ( μg/ml) | Potentiaiton |
|---|---|---|---|---|
| MCF7-sensitive | Doxorubicin | 0.2±0.05 | 0.04±0.01 | 5 |
| MCF7-sensitive | Etoposide | 20.9±2.4 | 0.08±0.03 | 261 |
| MCF7-sensitive | Paclitaxel | 0.18±0.08 | 0.002±0.001 | 90 |
| MCF7-resistant | Doxorubicin | 26.4± 7.4 | 1.6±0.6 | 16.5 |
| MCF7-resistant | Etoposide | 61.1±101 | 10.9±1.0 | 5.6 |
| MCF7-resistant | Paclitaxel | 4.2±0.6 | 1.5±0.6 | 2.8 |

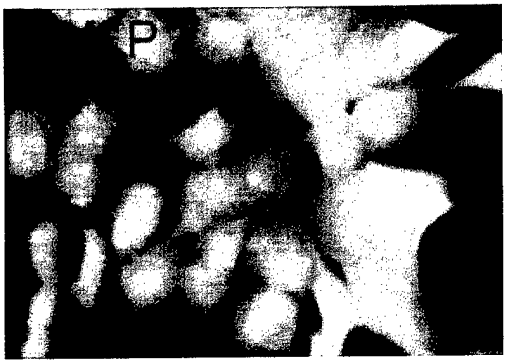
Potentaition= $IC_{50}$ for drug alone/ $IC_{50}$ for drug with monensin liposomes (SML).
Monensin liposomes (SML) were employed at $10x10^{-8}$ M and $20x10^{-8}$ M in MCF-7 sensitive and resistant cells, respectively.

**Effect of SML on the induction of apoptosis in resistant MCF-7 cells:** The induction of apoptosis in resitant MCF-7 cells by the combination of doxorubicin (at considerably much lower than its $IC_{50}$ value) with non-toxic concentration of SML ($20x10^{-8}$ M) was studied by acridine orange staining and caspase-3 assay. The combination of doxorubicin (as low as 0.5 μg/ml) with SML induced apoptosis in at least 30% of cells in comparison to less than 10% apoptotic cells observed in control, doxorubicin or SML treated cells (Figure 2). Further, the specific caspase-3 activity was enhanced by two-fold by the combination of doxorubicin (2.5 μg/ml) with SML, in comparison to that observed with doxorubicin alone. The results are shown in Figure 3.

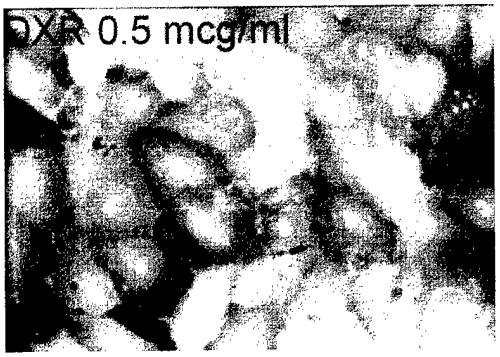
Control-MCF-7/dox cells




SML treated MCF-7/dox cells

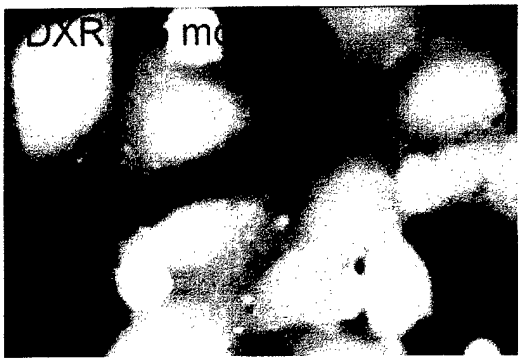



DOX (0.5 μg/ml) treated MCF-7/dox cells




DOX(0.5 μg/ml)+SML treated MCF-7/dox cells

DOX (2.5 μg/ml) treated MCF-7/dox cells



DOX (2.5 μg/ml)+SML treated MCF-7/dox cells

Figure 2: Induction of apoptosis in MCF-7/dox cells by the combination of doxorubicin (Dox) with SML.

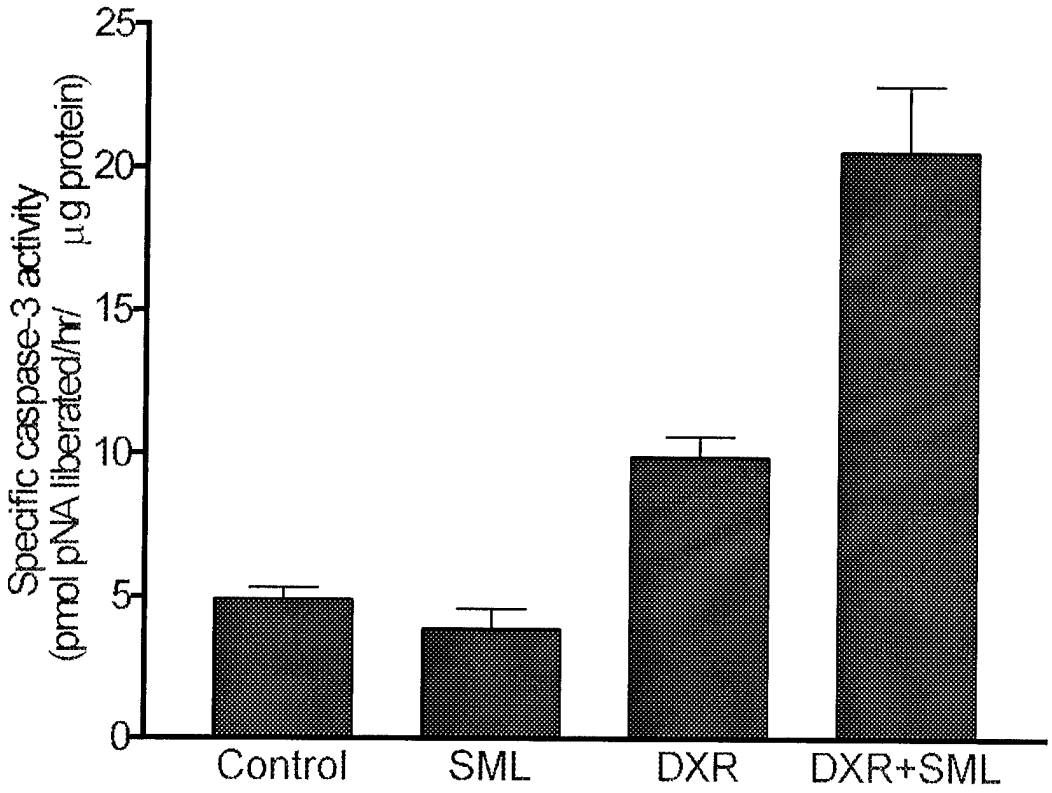



Figure 3: Enhancement of capspase-3 acitivity by the combination of doxorubicin (2.5 µg/ml) with SML ($20x10^{-8}$ M) against resistant MCF-7 cells.

Presently, studies are in progress to determine the molecular mechanisms associated with the reversal of doxorubicin resistance by SML in resistant MCF-7 cells. Further, the in-vivo studies with breast tumor bearing (MCF-7 sensitive and resistant) nude mice are being planned at the Collaborating Investigator's laboratory at the School of Medicine, Tulane University, New Orleans, LA.

## KEY RESEARCH ACCOMPLISHMENTS

1. The entrapment efficiency of monensin in SML was increased from 2% to 14% by employing pH-gradient method.

2. The SML was successfully freeze-dried with minimal change in particle size upon freeze-drying and subsequent storage, thus improving the physical stability of SML.

3. SML prepared by pH-gradient method were found to overcome the doxorubicin resistance in MCF-7/dox cells.

4. Apoptosis could be induced in MCF-7/dox cells by using non-toxic concentrations of SML with 1/50 th $IC_{50}$ concentration of doxorubicin.

## REPORTABLE OUTCOMES

1. **Mandip Singh**, MS Shaik, K. Primus, GA. Salama. Enhancement of the in-vitro cytotoxicity of anticancer drugs against sensitive and resistant human breast tumor MCF7 cells by stealth monensin liposomes. Presented at the 93rd Annual Meeting of the American Association for Cancer Research (AACR) held at San Francisco, April, 2002.

2. **Mandip Singh**, MS Shaik, K. Primus, GA. Salama. Enhancement of the in-vitro cytotoxicity and apoptotic response of doxorubicin in resistant MCF-7 cells by stealth monensin liposomes. To be presented at the Era of Hope 2002, Department of Defense Breast Cancer Research Program Meeting.

3. **Mandip Singh**, MS Shaik, K. Primus, GA. Salama. Enhancement of the in-vitro cytotoxicity and apoptotic response in doxorubicin resistant MCF-7 cells by stealth monensin liposomes. (Manuscript under preparation).

4. Two different doxorubicin resistant human breast tumor MCF-7 cell lines were developed.

## CONCLUSIONS

Stealth monensin liposomes enhance the in-vitro cytotoxicity of anticancer drugs against MCF-7 cells and overcome the doxorubicin resistance in the MCF-7/dox cells. Further, stealth monensin liposomes also enhance the apoptotic response of doxorubicin at much lower concentrations.

## REFERENCES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2. Mandip Singh, A. Ferdous and G. Faulkner. Stealth monensin immunoliposomes as potentiator of immunotoxins. European Journal of Pharmaceutics and Biopharmaceutics. 52: 13-20, 2001.

3. Madhu Sudhan Shaik, Narayanasamy Kanikkannan and Mandip Singh. Conjugation of anti-My9 antibody to stealth monensin liposomes and the effect of conjugated liposomes on the cytotoxicity of immunotoxin. Journal of Controlled Release. 76: 285-295, 2001.

induced by cerulenin on anthracycline toxicity were determined by using sensitization ratios ($IC_{30,50}$ DNR control/$IC_{30,50}$ DNR-cerulenin treated cells). Our data show that cerulenin induces a dramatic increase of DNR cytoxicity in MCF-7/Adr cells (up to 136-fold), and a significant increment in MCF-7 and SK-Br3 cells (up to 13 and 26-fold, respectively). To analyze whether the interaction between cerulenin and DNR was synergistic, additive or antagonistic, we used the isobologram method. A strong synergism in the three schedules was found in MCF-7/Adr cells, with combination indexes ranging from 0.283 to 0.376. In MCF-7 cells, the simultaneous schedule demonstrated synergism, whereas the sequential schedule was additive. In SK-Br3 cells, all combinations showed additivity. These results suggest a differential synergistic interaction between cerulenin and DNR among anthracycline-resistant or anthracycline-sensitive cells. When we used cerulenin as a single agent, MCF-7/Adr cells were 2-fold more resistant to cerulenin than MCF-7 cells, and 5-fold than SK-Br3 cells. This differential effect of cerulenin in MCF-7/Adr cells was reversed in the presence of Verapamil, a blocker of P-glycoprotein. To test the specifity of the synergism between DNR and cerulenin, we monitored the effects of palmitate, the metabolic end product of FAS. The simultaneous presence of palmitate with cerulenin and DNR reversed the potentiating effect of cerulenin on DNR toxicity. This suggests that the synergistic effects between cerulenin and DNR result from FAS product depletion. We also assessed the levels of $p21^{WAF1}$, a critical mediator of the cellular response to DNA damage. The $p21^{WAF1}$ levels were upregulated after DNR or cerulenin treatment alone. In contrast, the combination of cerulenin and DNR induced a marked downregulation of $p21^{WAF1}$, below the constitutive levels. This dysregulation of $p21^{WAF1}$ may be a critical molecular event mediating the synergism between DNR and cerulenin in MCF-7/Adr cells. In summary, anthracycline resistance can be reversed by the inhibition of FAS. This may be related to a disregulation of $p21^{WAF1}$, and provides a novel approach to overcome anthracycline resistance in breast cancer.

**#4710 Enhancement of the in-vitro cytotoxicity of anticancer drugs against sensitive and resistant human breast tumor MCF7 cells by stealth monensin liposomes.** Mandip S. Sachdeva, Madhu S. Shaik, Kela Primus, and Germain A. Salama. *Florida A&M University, Tallahassee, FL.*

Our laboratory has developed a method for improving the entrapment of monensin in stealth (long-circulating) liposomes by using pH-gradient approach. The stealth monensin liposomes have a particle size of 223 nm, monensin entrapment of 14%, and plasma half-life of 7-8 hr in BALB/c mice. In the present study, the liposomal monensin formulation was studied for its ability to enhance the in-vitro cytotoxicity of doxorubicin (DXR), etoposide (ETP) and paclitaxel (PTX) against both sensitive and resistant MCF7 cells. The cytotoxicity of DXR, ETP and PTX alone and in combination with stealth monensin liposomes was assessed by crystal violet assay. Furthermore, the induction of apoptosis in resistant MCF7 cells by DXR (at 1/50 th $IC_{50}$ concentration) in combination with stealth monensin liposomes was evaluated by acridine orange staining. Our results show that $IC_{50}$ of DXR, ETP and PTX against sensitive MCF7 cells was 0.2 $\mu$g/ml, 21.0 $\mu$g/ml and 0.1 $\mu$g/ml, respectively. The combination of liposomal monensin ($10x10^{-8}$ M) with DXR, ETP and PTX produced $IC_{50}$ values of 0.04 $\mu$g/ml, 0.1 $\mu$g/ml and 0.002 $\mu$g/ml against sensitive MCF7 cells, respectively (a 5, 210 and 50-fold potentiation of DXR, ETP and PTX, respectively). In case of resistant MCF7 cells, the $IC_{50}$ of DXR, ETP, PTX was found to be 26.5 $\mu$g/ml, 68.0 $\mu$g/ml and 5.0 $\mu$g/ml, respectively and their combination with liposomal monensin ($20x10^{-8}$ M) resulted in $IC_{50}$ values of 1.6 $\mu$g/ml, 10.9 $\mu$g/ml and 1.4 $\mu$g/ml, respectively (a 16.5, 6 and 3.5-fold potentiation of DXR, ETP and PTX, respectively). Acridine orange staining with resistant MCF 7 cells indicated that apoptosis was induced in at least 30% of cells by using DXR (0.5 $\mu$g/ml) and liposomal monensin ($20x10^{-8}$ M), as compared to the less than 10% in control, DXR and liposomal monensin treated cells. Our results indicate that it is possible to enhance the cytotoxicity of anticancer drugs like DXR, ETP and PTX by liposomal monensin in both sensitive and resistant MCF7 cells, which may be explored further in cancer chemotherapy.

**#4711 Decreasing levels of external oxygen increases tumor cell sensitivity to 2-Deoxy-D-Glucose: A strategy for solid tumor therapy.** Huaping Liu, Niramol Savaraj, Waldemar Priebe, and Theodore J. Lampidis. *University of Miami, School of Medicine, Miami, FL, and UT M.D. Anderson Cancer Ctr., Houston, TX.*

Previously we reported that two distinct *in vitro* tumor cell models of hypoxia (A & B) are hypersensitive to glycolytic inhibitors such as 2-deoxy-D-glucose (2-dg) [Biochemistry 40: 5542-5547, 2001]. Model A is osteosarcoma cells (143B) treated with agents which interfere with oxidative phosphorylation (OxPhos) and Model B is $\rho$O cells, which due to their deficiency in mitochondrial DNA, cannot perform OxPhos. Extending these studies we report here on Model C, which are 143B cells grown under varying levels of external $O_2$ (0, 0.1, 0.5, 1.0, & 5 %). At all lowered levels of $O_2$ tested,143B cells are hypersensitive to 2-dg when compared with cells grown at normal $O_2$ levels. In contrast, 143 B cells under hypoxic or aerobic conditions, show equal sensitivity to the standard chemotherapeutic agent vinblastine. Furthermore, $\rho$O cells when treated under reduced $O_2$ amounts, displayed no hypersensitivity to 2-dg and in fact were slightly more resistant than under aerobic conditions. At 0 to 1% $O_2$ levels, untreated 143B cells display reduced growth but elevated lactic acid levels. The results with

Abstract to be presented at Era of Hope 2002, DOD Breast Cancer Research Meeting

# ENHANCEMENT OF THE IN-VITRO CYTOTOXICITY AND APOPTOTIC RESPONSE OF DOXORUBICIN IN RESISTANT MCF7 CELLS BY STEALTH MONENSIN LIPOSOMES

**Mandip Singh, Madhu Sudhan Shaik, Kela Primus, Germain A. Salama, KC Agrawal***

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The carboxylic ionophore, monensin has been shown to modulate the doxorubicin resistance in various tumor cell lines in-vitro. However, monensin needs to be formulated in suitable drug delivery system in order to overcome its unfavorable physical and pharmacokinetic properties. We previously developed monensin into long-circulating (stealth) liposomes and showed that it could enhance the in-vitro cytotoxicity of anticancer drugs. In order to increase the entrapment of monensin in liposomes, we modified our previous method by using pH-gradient technique. In the present study, we studied the potential of stealth monensin liposomes (prepared by pH-gradient method) for their effect on the in-vitro cytotoxicity of anticancer drugs (doxorubicin, etoposide, paclitaxel) against both sensitive and resistant human breast tumor MCF7 cells by crystal violet dye uptake assay. Further, the induction of apoptosis in resistant MCF7 cells by the combination of doxorubicin with stealth monensin liposomes was also assessed by acridine orange staining and caspase-3 assay. Our results show that stealth monensin liposomes (10x10-8 M) enhance the in-vitro cytotoxicity of doxorubicin, etoposide and paclitaxel against sensitive MCF7 cells by a factor of 5, 261 and 90, respectively. In case of resistant MCF7 cells, there was 16.5, 5.6 and 2.8- fold potentiaiton of the cytotoxicity of doxorubicin, etoposide and paclitaxel, respectively by monensin liposomes (20x10-8 M). There was an enhanced apoptotic response (30%) in resistant MCF7 cells treated with doxorubicin at 0.5 mcg/ml (1/50 th IC50 concentration for doxorubicin) with nontoxic concentration of monensin liposomes (20x10-8 M) in comparison to less than 10% apoptotic response observed in control, doxorubicin and liposomal monensin treated cells. The specific activity of caspase-3 in resistant MCF7 cells treated with doxorubicin (2.5 mcg/ml) and monensin liposomes (20x10-8 M) was two times more than that of the cells treated with doxorubicin alone. The results indicate that it is possible to overcome the doxorubicin resistance in MCF7 cells with liposomal monensin, which may be further explored in-vivo in nude mice with human breast tumor xenografts.

Supported by the U.S. Army Medical and Material Command under DAMD17-00-01-0242.